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Cross-Linking Study on Skeletal Muscle Actin: Separation of CNBr Peptides of Suberimide-Treated Actin*

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Suberimide-treated F-actin, which contained about one mole of intramolecular cross-linkage per mole of actin, was cleaved with CNBr and the CNBr peptides were separated by column chromatography. The results suggest that intramolecular cross-linkage is incorporated in CB-16 (Lys-325, Lys-327, and Lys-335, nomenclature of CNBr-cleaved peptides is due to M. Elzinga *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 2687-2691 (1973)) or CB-17 (Lys-191, Lys-213, and Lys-215).

KEY WORDS: Skeletal Muscle Actin/ Cross-Linking/ Dimethylsuberimide/ CNBr Cleavage/ Actin Polymerization/

I. INTRODUCTION

Actin, one of the major proteins of the thin filament in skeletal muscle, is a globular protein with molecular weight of 42,000. Actin can take two states, a dispersed monomeric state (G-actin) and a filamentous polymeric state (F-actin), in response to the solvent conditions.¹⁾ Recently we reported that the cross-linking of F-actin with dimethylsuberimide affects the polymerization-depolymerization properties.²⁾ The suberimide-treated F-actin (SA) takes a filamentous form under the depolymerizing conditions for intact actin. We infer that the intramolecular cross-linking introduced in actin stabilizes the actin conformation in the F-state.

SA is appropriate for investigation of the mechanism of G-F transformation on the basis of the primary structure of actin. The determination of the location of cross-link introduced with suberimide in F-actin would elucidate how the cross-linkage between lysyl residues in actin restricts its conformational state. Since we developed the analytical method of suberimide-modified lysine on an amino acid analyzer,³⁾ the determination of the cross-linked site in SA is now possible. For this final goal, we adopted CNBr cleavage of SA followed by the separation of CNBr peptides. In this study, we report the separation of CNBr peptides of SA and the probable candidates of the cross-linked site.

Recently, actin is widely found in nonmuscle cells. In nonmuscle cells, actin is assumed to play an important roles in many cellular events. Therefore, the understanding of the mechanism of G-F transformation of actin is very important.

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Abbreviations: SDS, sodium dodecylsulfate; SA, suberimide-treated actin.

The cross-linking study would be one of promising ways to throw light on the conformational change during G-F transformation on the basis of the primary structure of actin.

II. EXPERIMENTAL PROCEDURES

1. Preparation of Actin and Suberimidate-Treated Actin (SA)

Actin was prepared from the acetone-dried powder of rabbit skeletal muscle according to the method of Spudich and Watt.⁴⁾ F-actin was treated with dimethyl-suberimidate as reported previously.²⁾ Protein concentration was determined by the ultraviolet absorption, $A_{1\%, 290\text{nm}} = 6.3$ for actin. The intermolecularly cross-linked dimer of actin, less than 15% of the modified protein, was removed by gel filtration on a column of Sephacryl S-300 (Pharmacia) equilibrated with 6 M urea-0.1 M NaCl-0.1 M acetic acid. Before the chromatography, cysteines in SA were carboxymethylated with iodoacetic acid.

2. CNBr Cleavage of SA and Separation of CNBr Peptides

The carboxymethylated SA, which contained only intramolecularly cross-linked species, was treated with CNBr according to the method of Lu and Szilagyi.⁵⁾ The entire mixture of CNBr peptides was chromatographed on a Sephadex G-50 column equilibrated with 25% acetic acid. The pooled fraction which contained cross-linked lysyl residues was lyophilized and further separated on a column of Sephadex G-10 equilibrated with 10 mM pyridine-acetic acid (pH 6.0) as described by Lu and Szilagyi.⁵⁾ The elution patterns of each column were monitored by absorption at 280 nm and/or ninhydrin staining after alkaline hydrolysis.

3. Analysis of The Peptides

The amino acid composition of the peptides were analyzed on a JEOL JLC-3 chromatographic system equipped with a JEOL LCR-BC₂ optical detection system, after hydrolysis in a sealed and evacuated tube with constant-boiling HCl at 110°C. The amount of the suberimidate-modified lysine was determined as reported.³⁾

4. Other Procedures

SDS-gel electrophoresis was performed in a system of Laemmli.⁶⁾ Dimethyl-suberimidate was prepared by the method of McElvain and Schroeder.⁷⁾

III. RESULTS

1. The Purification of Intramolecularly Cross-Linked SA Molecule

The modification with dimethylsuberimidate of F-actin produced a small amount of cross-linked dimer, which would disturb the determination of location of an intramolecular cross-link in an SA molecule. Therefore, the SA monomer was separated from the cross-linked dimer on a column of Sephacryl S-300 equilibrated with 6 M urea-0.1 M NaCl-0.1 M acetic acid as shown in Fig. 1. SDS-gel electrophoresis revealed that the pooled fraction I contained cross-linked dimer and SA monomer. After denaturation, the cross-linked dimer and SA monomer appeared to form a

Intramolecular Cross-link of Actin

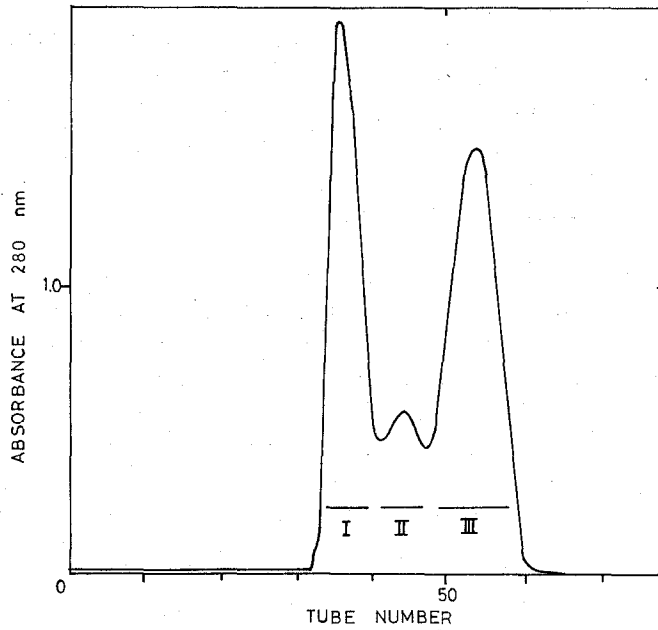


Fig. 1. Removal of cross-linked dimer from SA preparation.
The carboxymethylated SA (about 100 mg) was chromatographed on a column of Sephacryl S-300 equilibrated with 6 M urea-0.1 M NaCl-0.1 M acetic acid. Fractions were pooled as indicated.

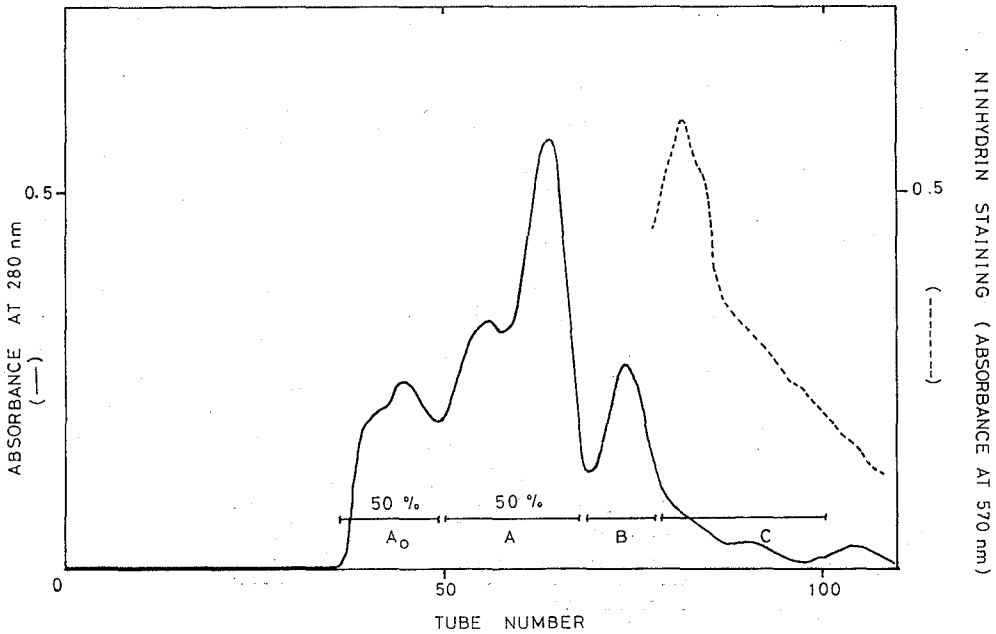


Fig. 2. Gel filtration of the entire mixture of CNBr peptides of SA on a Sephadex G-50 column equilibrated with 25% acetic acid.
The eluate of the column was monitored by absorption at 280 nm and ninhydrin staining after hydrolysis with NaOH. The amount of cross-linked lysine found in the pools, A₀ and A, are shown as % of the total amount in the figure.

large aggregate even in the presence of 6 M urea. Thus, if necessary, SA monomer could be recovered from the pooled fraction I by rechromatography on a Sephacryl S-300 column. Amino acid analysis indicated that SA monomer in the fraction III contained about one mole of cross-linked lysine and three mole of monofunctionally modified lysine per mole of actin molecule. Thus, the carboxymethylated SA monomer in the fraction III was used for further analysis.

2. The Separation of CNBr Peptides of SA

SA monomer was cleaved with CNBr, and the CNBr peptides were separated according to the strategy of previous work.^{5,8,9,10)} Following CNBr cleavage of SA, the entire mixture was chromatographed on a Sephadex G-50 and fractions A₀-C were pooled as shown in Fig. 2. Amino acid analysis showed that the pool A₀ and A contained cross-linked CNBr peptides. The peptides in the pool A were dissolved in 70% formic acid, and chromatographed on a column of Sephadex G-10. The pool A was resolved into two fractions, AA and AB, as shown in Fig. 3. Cross-linked lysine was found only in the acid hydrolysate of the peptides in the pool AB.

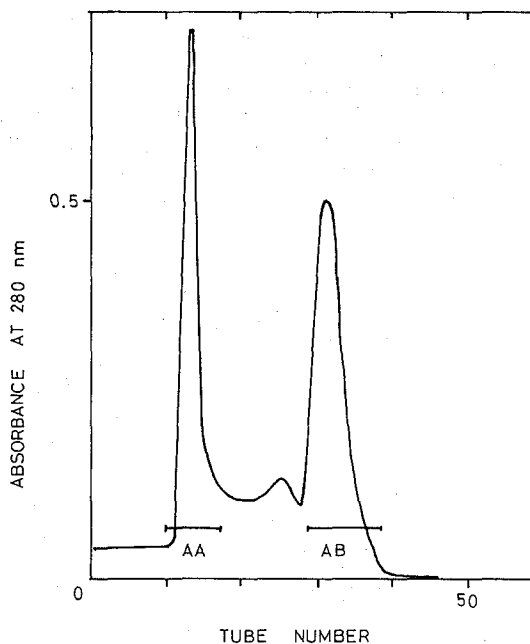


Fig. 3. Separation of peptides in the pool A on a column of Sephadex G-10 equilibrated with 10 mM pyridine-acetic acid (pH 6.0).

The peptides in the pool A were separated on the basis of their solubility in 10 mM pyridine-acetic acid, pH 6.0.

IV. DISCUSSION

After the removal of cross-linked dimer, SA was found to contain about one intramolecularly cross-linked and three monofunctionally modified lysyl residues.

In this study, we intended to locate cross-link incorporated in an actin molecule on the basis of its primary structure. For this final goal, we cleaved SA with CNBr and separated their CNBr peptides, since a scheme has already been developed to isolate the lysine-containing CNBr peptides by previous work.^{5,8,9,10} First, the entire mixture of CNBr peptides were separated according to their size by gel filtration on a Sephadex G-50 column. It was found that the gel filtration pattern of CNBr peptides of SA was the same as that of actin without a cross-link, suggesting that interpeptide cross-link was not formed: if the peptides were cross-linked together, the elution profile would be different from that of actin without a cross-link. Amino acid analysis shows that the cross-linked peptides were eluted in the pool A₀ and A. We cannot rule out the possibility that the peptides in the pool A₀ contain the interpeptide cross-link. However, the amount of peptides in the pool A₀ was varied from preparation to preparation and we confirmed that the pool A₀ contained the partially cleaved CNBr peptides. Since separation of the partially cleaved CNBr peptides could not be carried out with the strategy developed previously, analysis of the peptides in the pool A₀ would be very complicated. Therefore, we analyzed the peptides in the pool A to determine the cross-linked site. The peptides in the pool A were separated into two fractions according to their solubility of the peptides in 10 mM pyridine-acetic acid, pH 6.0. Amino acid analysis revealed that cross-linked lysine was found in the acid hydrolyzate of the peptides in the fraction AB, which is less soluble than those in the fraction AA in pyridine-acetic acid buffer.

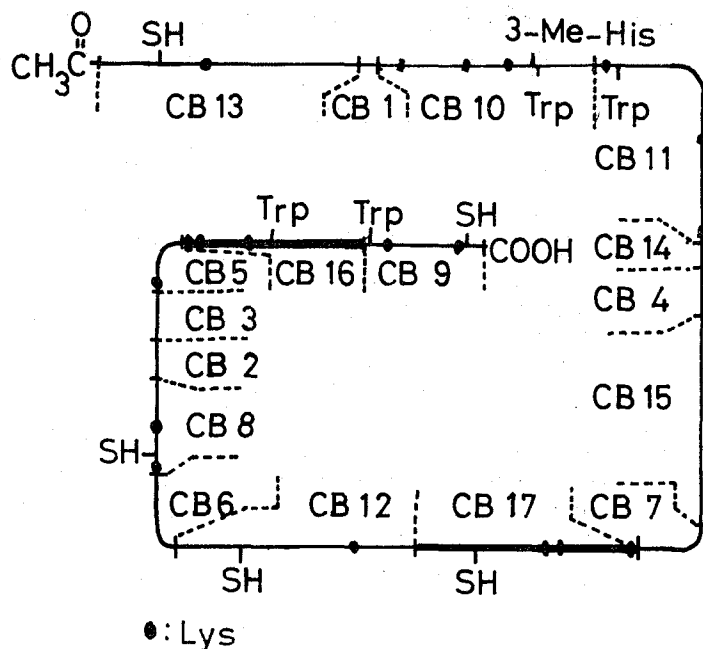


Fig. 4. Distribution of lysyl residues in primary structure of actin.

The location of CNBr peptides (from CB-1 to CB-17) is also illustrated. The filled circles show the position of lysyl residues and the thick lines indicate the position of CB-16 and CB-17.

According to the previous work, the fraction AB must contain the lysine-containing peptides, CB-16 and CB-17, designated in the previous work. Thus, the intramolecular cross-link was formed between lysyl residues in CB-16 (Lys-325, Lys-327, and Lys-335) or CB-17 (Lys-191, Lys-213, and Lys-215). The distribution of lysyl residues and CNBr peptides designation are illustrated in Fig.4.

We assigned the cross-linked site in an SA molecule tentatively as described above. If this assignment is correct, the conformation of actin in the F-state is stabilized by the introduction of cross-link between near lysyl residues in its primary structure. That is, G-F transformation might require only a subtle conformational change. Recently, Sutoh reported that intermolecular cross-link was formed between Cys-373 and a lysyl residue in CB-17, indicating that CB-17 are close to the actin-actin contact sites in a double helical polymer of F-actin.¹¹⁾ Furthermore, Lu and Szilagyi demonstrated that lysyl residues in CB-16 and CB-17 are moderately reactive to reductive methylation and that the reactivity of Lys-335 in CB-16 is strikingly enhanced.⁵⁾ These results support our assignment of the intramolecularly cross-linked site of SA.

We cannot exclude the possibility that the cross-linked site is not unique: SA may not be homogeneous in terms of the intramolecularly cross-linked site. In this study, we failed the isolation of the CNBr peptides which contained the cross-link due to the loss of the peptides in the pool AB during further separation. The amount of starting material of SA should be increased for the quantitative isolation of the cross-linked CNBr peptide. Therefore, our assignment of the cross-linked site is only tentative at the present stage and the cross-linked site will be conclusively determined only after the quantitative isolation of a cross-linked peptide of SA.

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